Measurement of Oxygen Solubility in Fermentation Media: A Colorimetric Method

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Methods of measuring oxygen solubility in culture media are scarce, and those available are tedious to apply. A simple colorimetric assay was developed and applied to the analysis of oxygen solubility during alcoholic fermentation. The method was based on the consumption of oxygen by glucose oxidase activity and the production of the pink quinone of syringaldazine by coupled peroxidase activity. Color formation at 526 nm progressed through an optimum that was a linear function of the oxygen added to the assay. Sensitivity was maximized by operating at pH 7 and limiting the medium sample volume added. Each assay took 10–15 min to prepare and react. Reaction time was minimized by using abundant glucose and enzyme concentrations. Data obtained by the assay developed showed good agreement with published oxygen solubilities in water and selected media at various temperatures. Subsequent analyses of fermentation broths indicated falling sugar concentration to be primarily responsible for increases in oxygen solubility during fermentation. For example, during fermentations started with 230 g/L xylose or glucose, oxygen solubility could increase by 41% due to sugar consumption alone. This procedure can provide the solubility data needed to accurately calibrate in-line electronic probes for monitoring dissolved oxygen concentration during fermentation processes.

INTRODUCTION

Dissolved oxygen concentration is a critical factor that often regulates fermentation rate and yield. It can be monitored on-line via an electrode sensitive to oxygen tension. Following sterilization *in situ*, the electrode is calibrated by setting zero against nitrogen and full scale against air-equilibrated medium. Thus, data are in terms of relative fraction saturation (f). The oxygen solubility (C_{ox}^{*}) of the medium must be known in order to calculate concentration $C_{ox}(= fC_{ox}^{*})$. Winkler's method[1] and NADH oxidation by mitochondria[2,3] or phenazonium methosulfate[4] are direct chemical measures commonly used to provide solubility data. Winkler's method is suitable for testing dilute aqueous solutions, but it fails in the presence of certain ingredients at concentrations commonly found in culture media. NADH oxidation has potential for application, but it is tedious and has been tested on phosphate buffers only, not on complete media. Thus despite mechanical complexity, manometry has been preferred for determining gas solubilities in fermentation broths.[5]

A colorimetric assay for C_{ox}^{*} is suggested by the following reactions:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \underset{\text{(colorless)}}{\text{syringaldazine}} \xrightarrow{\text{peroxidase}} \underset{\text{(pink at 526 nm)}}{\text{syringaldazine quinone}}$$

Syringaldazine quinone (tetramethoxy-azo-bis-methylene-quinone) has a molar absorption coefficient of 6.5×10^4 at 526 nm in 50% aqueous methanol.[6] Being highly colored, it is a sensitive chromagen allowing detection of low oxygen concentrations if coupled with peroxidase and glucose oxidase reactions. Sensitivity is critical when evaluating oxygen solubility of culture media because samples must be diluted in the assay mixture to prevent interference of media ingredients with enzyme activities. The chemistry of the chromagen reaction has been described in detail by Harkin and Obst.[6]

Based on these principles, a colorimetric assay was developed to measure oxygen solubility in culture media. All reactants, except for oxygen, were injected into septum-capped cuvettes under a nitrogen sparge. The assay was initiated by injecting oxygen-equilibrated medium or water, and reaction progress was followed by recording absorbance (A) at 526 nm. Oxygen-saturated water at known

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temperature (T) and pressure (P) served as standard for the assay since published solubility data were readily available.[7] Experiments optimized assay conditions (pH, medium sample size, reagent concentrations), correlated maximum color formation with dissolved oxygen concentration, determined assay linearity, and demonstrated agreement of assayed C_{ox}^{*} with published data. Finally, the newly developed assay was applied to evaluate C_{ox}^{*} for various culture media.

MATERIALS AND METHODS

Culture Media

During assay development, we used CCY as a model fermentation medium. It was a rich solution composed of (per liter) 10 g yeast extract, 0.011 g CaO, 0.004 g ZnO, 0.054 g $FeCl_3 \cdot 6H_2O$, 0.0036 g MgO, 0.0025 g $CuSO_4 \cdot 5H_2O$, 0.0024 g $CoCl_2 \cdot 6H_2O$, 0.006 g H_3BO_3, 0.101 g MgO, 6.4 g urea, 1.2 g KH_2PO_4, and 0.18 g Na_2HPO_4. Xylose was autoclaved as a separate solution to give the completed medium a concentration of 50 g/L, unless otherwise specified. The final pH was adjusted to 4.5 with 2N HCl. Where indicated, Hodag FD-62 antifoam was added to a concentration of 1 g/L completed medium. Further details about media preparation are given by Slininger et al.[8]

Other media tested for oxygen solubility included MRS, TGY, TSB, YM, and YNB. MRS medium contained (per liter) 10 g Difco proteose peptone No. 3, 10 g Difco beef extract, 5 g Difco yeast extract, 2 g K_2HPO_4, 2 g ammonium citrate [$(NH_4)_2HC_6H_5O_7$], 5 g sodium acetate ($NaC_2H_3O_2$), 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot H_2O$, and 1.2 g Tween 80 [or MCB Reagents' polyoxyethylene (20) sorbitan monooleate]. TGY contained 5 g Difco tryptone, 5 g yeast extract, and 1 g KH_2PO_4 per liter. TSB was 30 g/L Trypticase Soy Broth prepared medium (BBL Microbiology Systems product 11774). YM consisted of (per liter) 3 g each of Difco yeast and malt extracts, and 5 g Difco peptone. YNB was 6.7 g/L dehydrated Difco Yeast Nitrogen Base. As appropriate, each medium was either nitrogen- or oxygen-sparged at least 15 min.

Assay Procedure

Table I summarizes the suggested procedure. Enzyme solution was prepared by diluting 1000 units of glucose oxidase and 200 units of peroxidase (Sigma products G-6500 and P-8375, respectively) to 100 mL with 0.1M phosphate buffer (pH 5–7.5, as specified). It was cooled on ice under nitrogen but continual sparging was avoided to prevent loss of enzyme activity. Assays were carried out at ambient temperature and pressure in septum-capped glass spectrophotometer cells with 1 cm path length (Thomas Scientific No. 8493-D12 and -D29). Such cuvettes permitted injection of assay ingredients while a nitrogen sparge maintained oxygen-free conditions. Bottled nitrogen (Amerigas) was used without removing trace oxygen because corrections for background oxygen were small enough to subtract without sacrificing accuracy. The sparge was a 2-in., 18-gauge needle inserted through the cap to bubble gas at the base of the cuvette. A 22-gauge needle just piercing the cap served as the vent. Assays were initiated by injecting media or water equilibrated with pure oxygen at known temperature and pressure. Equilibration was achieved by bubbling oxygen ca. 15 min in loosely covered tubes. An oxygen electrode was used to check the bubbling time needed. Reactions were monitored with a Bausch & Lomb Spectronic 2000 recording spectrophotometer until color reached its maximum A'_{max} at time t_{max}. The following calculation was applied to correct for background absorbance (A_b) introduced for example by trace oxygen in the nitrogen stream: $A_{max} = A'_{max} - A_b$. Trace oxygen normally contributes ≤ 0.02 absorbance units. Medium coloration can potentially contribute to the background absorbance, but sample dilution in the assay generally eliminates the need for a medium correction. After ca. six reactions, cuvettes and caps were soaked in warm Haemo-Sol to remove protein deposits inhibitory to enzymes. Otherwise cuvettes were rinsed with distilled water between reactions.

Table I. Summary of suggested assay for oxygen solubility.

Procedure	Ingredient volume added (mL): Reference water assay	Ingredient volume added (mL): Test medium assay
O_2-free reagent preparation		
1) Combine in a cuvette:		
enzyme solution (pH 7)	0.3	0.3
0.1M phosphate buffer (pH 7)[a]	0.2	0.2
H_2O[a]	2.8	2.82
1.6 g/L syringaldazine/methanol	0.01	0.01
2) Cap cuvette and sparge 2 min with N_2	—	—
3) Inject test medium[a]	0.02	0
4) Sparge 1 min with N_2	—	—
5) Inject 3 g/L glucose solution[a]	0.035	0.035
6) Sparge 1 min with N_2 then remove both sparge and vent	—	—
7) Measure background A_b (526 nm)	—	—
Reaction initiation		
8) Inject H_2O	0.02	0
or test medium equilibrated with pure O_2 at known T, P	0	0.02
9) Measure A_{max} and t_{max}	—	—
Oxygen solubility calculation[b]		
10) $C_{ox}^{*} = \left(\frac{A_{max,test}}{A_{max,ref}}\right) C_{ox,w}^{*}$	—	—

[a] Solution is bubbled with N_2 prior to use.

[b] $C_{ox,w}^{*}$ and C_{ox}^{*} are oxygen solubilities in water and medium, respectively, under pure oxygen at known T, P. The value of $C_{ox,w}^{*}$ is obtained from the literature.

RESULTS AND DISCUSSION

Optimization of Assay Conditions

Once each assay was initiated, color development passed through a maximum A_{max} at time t_{max} (Fig. 1). An uncertainty of ±0.02 absorbance units was observed. This behavior suggested competing reactions which produce and consume the colored quinone. Because the outcome of this competition is subject to conditions, A_{max} and t_{max} are likely to vary with pH, medium content, and concentrations of glucose, enzyme, and syringaldazine. Reference water assays (Table I) of varying composition were run to evaluate such effects. Each was initiated by injecting 0.02 mL water equilibrated with pure oxygen at 24°C and 762 mm Hg total pressure.

Color fading has been associated with pH-mediated addition of acid or alkali across the two methide structures of the quinone formed.[6] High protein concentrations interfere with the glucose oxidase/peroxidase reactions,[9] and it is conceivable that the degree of interference may change with pH because of induced changes in molecular charge. When evaluated as a function of both pH and CCY (protein) content (Fig. 2), A_{max} decreased with increasing CCY, but the curve shifted with changes in pH. The direction of the shift was upward as pH increased from 5 to 7, but downward as pH exceeded 7. At pH 7, A_{max} was greatest, and there was negligible decline in A_{max} with CCY contents to 0.02 mL per 3.3-mL assay volume. Neither pH or CCY variations affected t_{max}.

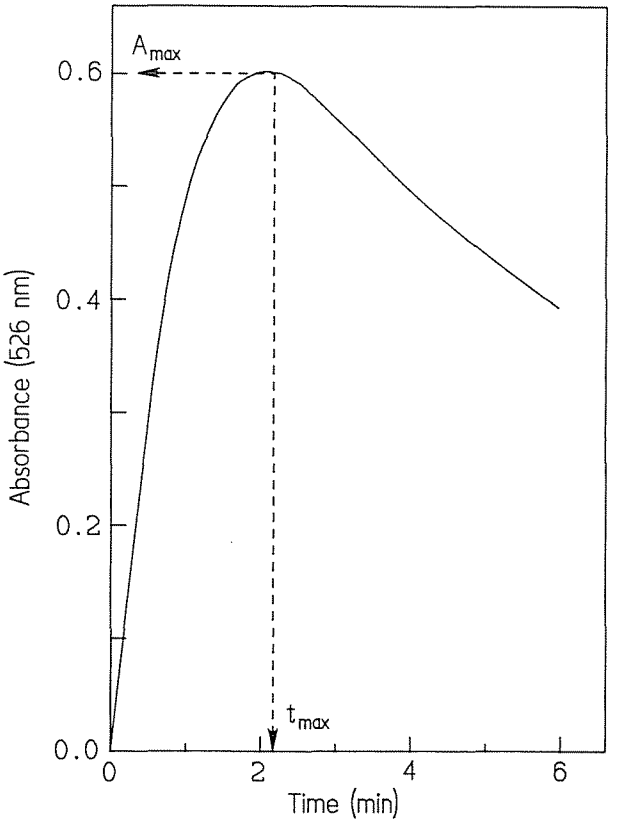

Figure 1. Time course of color formation at 526 nm following assay initiation with oxygenated water. The uncertainty in A_{max} was ±0.02 absorbance units.

Table II summarizes effects of enzyme, glucose, and syringaldazine concentrations on A_{max} and t_{max}. Parameter A_{max} was not very sensitive to changes in any of these components, but t_{max} decreased with increasing enzyme and glucose concentrations. Although a fast reaction saves time, there are sacrifices in cost and reproducibility which must be weighed when choosing reagent concentrations. Increases in syringaldazine concentration led to loss of reproducibility as its solubility was surpassed.

A_{max} and t_{max} Versus Oxygen Concentration

Reference water assays were initiated with 0–0.020 mL water equilibrated with pure oxygen at 24°C and 764 mm Hg total pressure. Figure 3 shows that A_{max} was linearly dependent on the volume of oxygen-saturated water added. Figure 3 also shows that t_{max} neared 2 min as the volume of oxygenated water approached 0.02 mL. Although this plot became linear as oxygen increased, it was nonlinear near the origin, assuming $t_{max} = 0$ in the absence of oxygen. Consequently, variation of reaction rate with oxygen concentration was implied.

Oxygen Solubility Versus Temperature: Experimental Versus Published Data

Published data[7] were used to calculate the fraction,

$$f_{20} = C^*_{ox,T}/C^*_{ox,20},$$

Where $C^*_{ox,T}$ and $C^*_{ox,20}$ refer to solubilities of oxygen (760 mm Hg) in water at temperature T and 20°C, respectively. Since A_{max} is proportional to C^*_{ox} (Fig. 3), the same fraction can be calculated from experimental A_{max} data as follows:

$$f_{20} = A_{max,T}/A_{max,20}$$

To gather experimental data for comparison with published data, a series of reference water assays containing CCY

Table II. Dependence of A_{max} and t_{max} on assay content of enzyme, glucose, and syringaldazine.[a]

Reagent varied	Reagent solution volume (mL)	A_{max}	t_{max} (min)
Enzyme	0.200	0.565	6.7
	0.300	0.598	4.3
	0.400	0.597	3.3
Glucose	0.015	0.565	6.7
	0.025	0.563	4.0
	0.035	0.600	3.2
Syringaldazine	0.010	0.565	6.7
	0.020	0.534[b]	6.4

[a] Assay compositions were as given for the reference water assay in Table I, except that 0.2 mL enzyme and 0.015 mL glucose solution were standardly used unless varied in experiments.

[b] Adding more than 0.010 mL syringaldazine solution led to high background absorbances and loss of reproducibility.

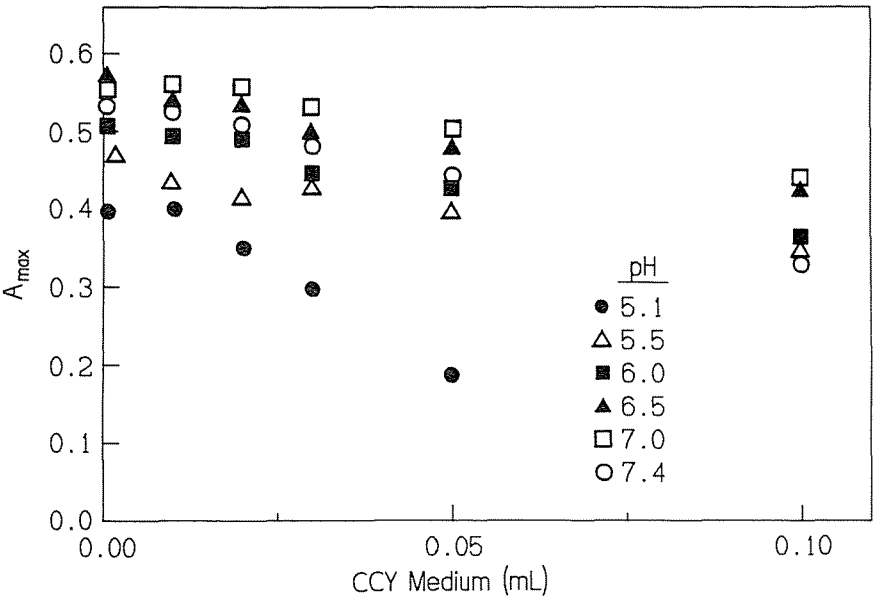

Figure 2. Variation of A_{max} with the presence of CCY medium in the assay buffered at different pH values.

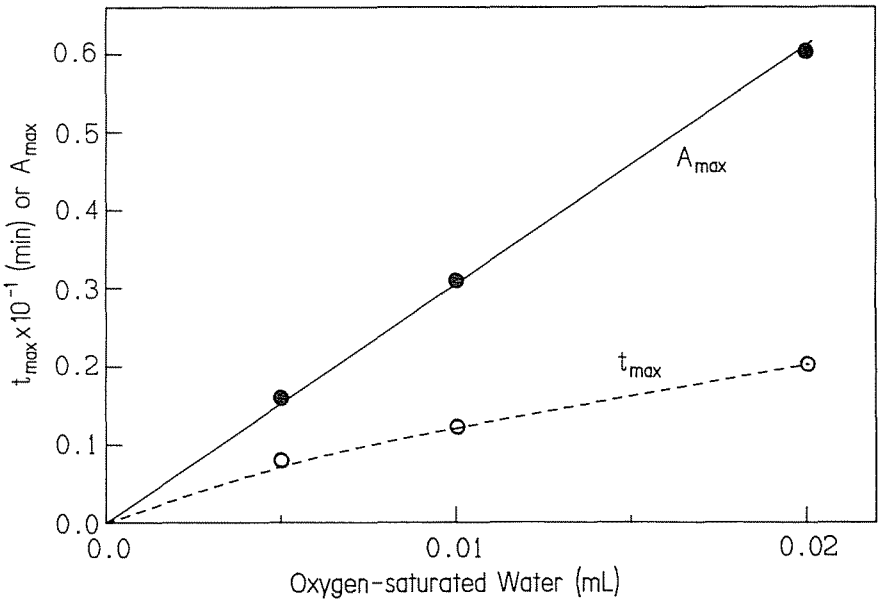

Figure 3. Dependence of A_{max} and t_{max} on the volume of oxygen-saturated water used to initiate the reference assay of Table I (CCY medium present).

medium were run as described in Table I. They were initiated with 0.020 mL water which was equilibrated with pure oxygen (764 mm Hg) at various temperatures (20–37°C). Figure 4 shows that plots of f_{20} vs. temperature for published and experimental data were in close agreement. This result confirms the validity of using published oxygen solubilities in water to calibrate the assay. It also suggests that diffusion of oxygen into the N_2 headspace in the reaction cuvette is insignificant.

Oxygen Solubilities in Culture Media

To control for media ingredient effects, both the reference water and test medium assays of Table I were performed to obtain $A_{max,ref}$ and $A_{max,test}$ for each culture medium. The ratio of $A_{max,test}$ to $A_{max,ref}$ gave oxygen solubility in the medium as a fraction of the solubility in water. Multiplying this ratio and a literature value for oxygen solubility in water $(C^*_{ox,w})$[7] yielded C^*_{ox} for the medium at the same values of T and P_{O_2}.

Table III shows oxygen solubilities measured on various culture media. Solutions of meat extract, peptone, proteose peptone No. 3, tryptone, and yeast extract, had solubilities similar to that of water. Handbook data[10] show that these media ingredients are composed primarily of peptides, amino acids, nucleic acids, and vitamins with small amounts of ash and salt. Media prepared without added sugar such as CCY, MRS, TGY, TSB, YM, and YNB

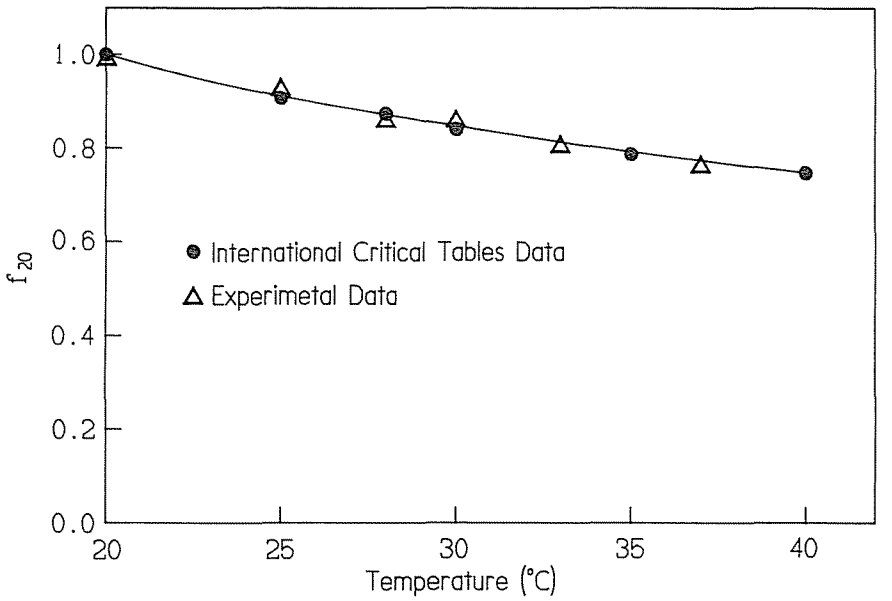

Figure 4. Comparison of $f_{20} = C^*_{ox,T}/C^*_{ox,20}$ as calculated from International Critical Tables data and $f_{20} = A_{max,T}/A_{max,20}$ as calculated from experimental data.

Table III. Effect of media composition on oxygen solubility (30°C, 760 mm Hg of O_2).

Medium	$A_{max,test}/A_{max,ref}$	C^*_{ox} (mg/L)[a]
Solutions of various ingredients		
Malt extract (10 g/L)	0.98	36.7
Meat extract (10 g/L)	1.00	37.4
Peptone (5 g/L)	1.01	37.8
Proteose peptone No. 3 (10 g/L)	0.98	36.7
Tryptone (5 g/L)	1.00	37.4
Yeast extract (10 g/L)	1.01	37.8
Complete media (no sugar added)		
CCY	0.94	35.2
MRS	0.88	32.9
TGY	0.96	35.9
TSB	0.92	34.4
YM	0.97	36.3
YNB	0.97	36.3
CCY with antifoam and ethanol		
CCY + 1 g/L Antifoam = CCYA	0.94	35.2
CCYA + 10 g/L ethanol	0.92	34.4
CCYA + 20 g/L ethanol	0.92	34.4
CCYA + 30 g/L ethanol	0.99	37.0
CCYA + 40 g/L ethanol	0.98	36.7
CCYA + 50 g/L ethanol	0.98	36.8
CCYA + 60 g/L ethanol	0.98	36.7
CCYA + 70 g/L ethanol	0.96	35.9

[a] $C^*_{ox} = (A_{max,test}/A_{max,ref})C^*_{ox,w}$, where $C^*_{ox,w}$ = 37.4 mg/L under 760 mm Hg of O_2 at 30°C[6]; C^*_{ox} is accurate to ±1 mg/L.

contained certain of these ingredients but showed solubilities 6–12% less than water, presumably because of added salts and buffers. The inclusion of 1 g/L antifoam did not change oxygen solubility in CCY medium.

During fermentation, medium contents vary considerably over time. Examples of interest are the conversions of glucose to ethanol and xylose to xylitol and ethanol. Table III and Figure 5 show the dependences of oxygen solubility on sugar and product concentrations which commonly occur during fermentation. Xylose and glucose at 230 g/L lowered oxygen solubility to 71% of that for the sugar-free medium. Xylitol at 40 g/L lowered oxygen solubility of the media by 9%. This is similar to the effect of 40 g/L xylose on oxygen solubility. However, xylose fermentations with potential for application produce less than 10 g/L xylitol, giving it only a minor role in determining oxygen solubility. A slight increase in oxygen solubility as ethanol concentration was increased from 0 to 70 g/L was observed. This is consistent with the fact that oxygen is more soluble in ethanol (204 mg/L under 760 mm Hg O_2 at 19.8°C) than in water.[7] Our analysis indicates that changes in sugar concentration are primarily responsible for changes in oxygen solubility during the course of alcoholic fermentation of glucose and xylose. Using data collected by manometry, Popovic et al.[11] arrived at similar results. Their solubility measurements on glucose and yeast extract solution differed from ours by less than 5%.

CONCLUSIONS

The glucose oxidase–peroxidase enzyme system with syringaldazine as chromagen is suitable for assaying oxygen solubility in complex media. The final quinone concentration, as indicated by A_{max}, is a linear function of oxygen added to the assay. Assay sensitivity (A_{max}) is optimum at pH 7, but depends on the type and volume of medium added. The reaction time (t_{max}) can be shortened to 2 min or less by raising the enzyme and/or glucose concentration used in the assay. Based on these findings an assay composition and procedure has been suggested (Table I). To control for media effects, it is recommended that the medium sample size be standardized and that both reference and sample assays be performed. Literature data

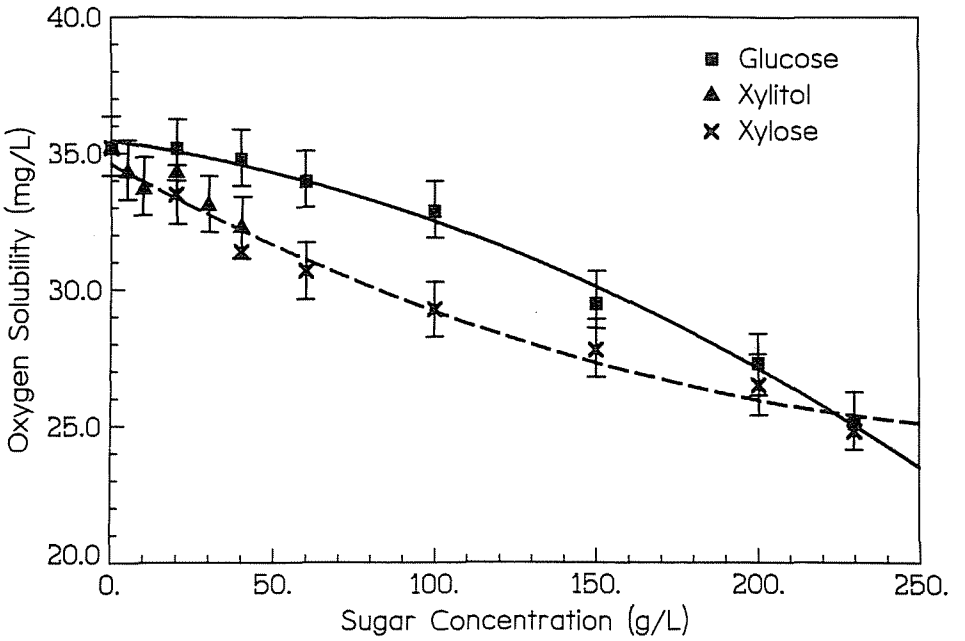

Figure 5. Dependence of oxygen solubility on sugar concentration (30°C, 760 mm Hg of O_2). Curves fitted to the data followed the equations $C_{ox}^{*} = 35.5 - 0.0170G - 0.000123G^2$ and $C_{ox}^{*} = 34.6 - 0.0644X + 0.000156X^2$ where G and X represent glucose and xylose (or xylitol) concentrations, respectively. As shown by error bars, the uncertainty in measured oxygen solubilities was ±1 mg/L.

on oxygen solubility in water at known temperature and pressure could be used to calibrate the assay. An example application of the assay in the analysis of glucose and xylose media suggests that sugar consumption is primarily responsible for rising oxygen solubility during alcoholic fermentation.

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